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**An IncP plasmid carrying the colistin resistance gene *mcr-1*  
in *Klebsiella pneumoniae* from hospital sewage**

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**Running title: *mcr-1* in sewage**

**Keywords:** colistin resistance; *mcr-1*; sewage; *Klebsiella pneumoniae*

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20   **Abstract**

21    A *Klebsiella pneumoniae* strain of ST313 recovered from hospital sewage was found  
22    to carry the plasmid-borne colistin resistance gene *mcr-1*, which was bracketed by two  
23    copies of the insertion sequence IS*Apl1* on a 57-kb self-transmissible IncP type  
24    plasmid of a new IncP-1 clade. The carriage of *mcr-1* on a self-transmissible  
25    broad-host-range plasmid highlights that *mcr-1* has the potential to be spread beyond  
26    the *Enterobacteriaceae*.

Colistin is the last resort antimicrobial agents to treat the infections caused by many Gram-negative bacteria. Recently, a plasmid-borne colistin resistance gene, *mcr-I*, has been found in *Escherichia coli* and *Klebsiella pneumonia* from human and animals in China (1). A few follow-up studies have found *mcr-I*-carrying *E. coli* in many countries in Africa (2), Europe (3-8), Asia (1-3, 9, 10) and North America (9, 11). In addition, *mcr-I* has also been found in *Kluyvera ascorbata* (12) and several species of *Salmonella* (13-15). All of above findings suggest that *mcr-I* has been widely distributed and imposes an emerging threat for clinical management and public and animal health. During a screening study for the presence of colistin-resistant *Enterobacteriaceae* in hospital sewage, we found a *mcr-I*-carrying *K. pneumonia*, which is reported here.

*K. pneumoniae* strain WCHKP1511 was recovered from the influx mainstream of hospital sewage at West China Hospital, Chengdu, western China, in November 2015. Strain WCHKP1511 grew on CHROMAgar Orientation agar plates (CHROMAgar, Paris, France) containing 4 µg/ml colistin and 64 µg/ml linezolid. Species identification was established by partially sequencing the *gyrB* gene (16). Strain WCHKP1511 was resistant to colistin (MIC, 8 µg/ml), polymyxin B (MIC, 8 µg/ml), chloramphenicol (MIC, 128 µg/ml) and tetracycline (MIC, 64 µg/ml) but was susceptible to amikacin (MIC, 0.5 µg/ml), ceftazidime (MIC, ≤0.5 µg/ml), cefotaxime (MIC, 0.03 µg/ml), ciprofloxacin (MIC, 0.03 µg/ml), imipenem (MIC, 0.125 µg/ml)

and tigecycline (MIC, 1 µg/ml) as determined using the microdilution broth method following recommendations of the Clinical Laboratory Standards Institute (CLSI) (17). WCHKP1511 was susceptible to fosfomycin (MIC, 32 µg/ml) as determined using the agar dilution method following recommendations of CLSI (17). In addition, strain WCHKP1511 was resistant to ampicillin and trimethoprim-sulphamethoxazole, intermediate to amoxicillin-clavuanate, gentamicin, tobramycin and nitrofurantoin, and susceptible to aztreonam, cefazolin, cefepime, cefoxitin, ceftriaxone, ertapenem, levofloxacin and piperacillin-tazobactam as determined using the Vitek II automated system (bioMerieux, Lyon, France). Breakpoints defined by FDA and by EUCAST were used for tigecycline and colistin, respectively; otherwise, those defined by CLSI were applied. Strain WCHKP1511 was found to carry *mcr-1* by PCR and sequencing (1, 12). Although *mcr-1* has been widely found in *E. coli*, *mcr-1*-carrying *K. pneumoniae* remains uncommon and had only been found in eastern China (Jiangsu and Zhejiang provinces) before (1, 18).

Strain WCHKP1511 was subjected to the 150-bp paired-end whole genome sequencing with a ca. 200× coverage using the HiSeq 2500 Sequencer (Illumina, San Diego, CA, USA). A total of 3,784,972 reads and 567,745,800 clean bases were generated, which were assembled into 275 contigs (230 contigs ≥ 1,000 bp) in length (N50, 67,217 bp) with a 57.46% GC content using the Spades program (19).

69 WCHKP1511 belonged to ST313, which was determined by using the genomic  
70 sequence to query the multi-locus sequence typing database of *K. pneumoniae*  
71 (<http://bigsdh.web.pasteur.fr/klebsiella/klebsiella.html>). In the *K. pneumoniae* MLST  
72 database, only one ST313 strain, KML 2185, which was recovered from human blood  
73 in the Netherlands in 2007, has been deposited. *K. pneumoniae* strains carrying *mcr-1*  
74 in Jiangsu province belonged to ST25 (20), while the STs of those in Zhejiang  
75 province were unknown. ST313 was not closely related to ST25, as only 2 out of 7  
76 alleles were identical between the two STs.

77

78 Antimicrobial resistance genes were predicted using ResFinder from the Center for  
79 Genomic Epidemiology (<http://genomicepidemiology.org/>). In addition to *mcr-1*,  
80 WCHKP1511 had multiple genes mediating resistance to aminoglycosides  
81 (*aac(3)-Iva*, *aadA2*, *aph(3')-Ia* and *aph(4)-I*),  $\beta$ -lactams (*bla*<sub>TEM-135</sub> and a new *bla*<sub>SHV</sub>  
82 variant), chloramphenicol (*floR*), fosfomycin (*fosA*), quinolones (*oqxA* and *oqxB*),  
83 sulphonamides (*sul2*), trimethoprim (*dfrA12*) and tetracycline (*tet(A)*) as predicted by  
84 ResFinder. *bla*<sub>TEM-135</sub> encodes a broad-spectrum  $\beta$ -lactamase (21). Of note, although  
85 strain WCHKP1511 carried *fosA*, it was susceptible to fosfomycin, which warrants  
86 further investigation. *bla*<sub>SHV</sub> in strain WCHKP1511 is a new variant, which encodes a  
87 SHV enzyme with an amino acid difference (Thr14Asn, the position is based on the  
88 ATG start condon) from SHV-111, the closest match. The new SHV has been assigned  
89 SHV-195 by the NCBI  $\beta$ -lactamase classification system

([www.ncbi.nlm.nih.gov/pathogens/submit\\_beta\\_lactamase/](http://www.ncbi.nlm.nih.gov/pathogens/submit_beta_lactamase/)). As strain WCHKP1511 was susceptible to third generation cephalosporins, *bla*<sub>SHV-195</sub> is unlikely to encode an extended-spectrum  $\beta$ -lactamase (ESBL). The complete coding sequence of *bla*<sub>SHV-195</sub> was cloned onto the pBC SK vector (Agilent, Santa Clara, CA, USA), which was electroporated into *E. coli* DH5 $\alpha$ . *E. coli* transformants containing *bla*<sub>SHV-195</sub> were resistant to ampicillin (MIC, >256  $\mu$ g/mL) and cephalothin (32  $\mu$ g/mL) but were susceptible to aztreonam, ceftazidime, cefotaxime, cefoxitin and imipenem determined using the broth microdilution method (17). This confirmed that *bla*<sub>SHV-195</sub> encodes a broad-spectrum rather than an ESBL.

In addition, there was a predicted bleomycin-resistance gene, designated ORFble here, which was not identified by Resfinder but was identified by the Prokka annotation tool (22) and was confirmed by Protein-BLAST in strain WCHKP1511. The complete coding sequence of ORFble was amplified with primers 1511\_ble\_BamHI\_F (CGCGGATCCTTGGTTCACCATGAAGATG)/1511\_ble\_EcoRI\_R (CCGGAATTCCGGCCGATTGCTGAACAGATTA), was cloned onto pBC SK and was electroporated into *E. coli* DH5 $\alpha$ . ORFble-containing transformants were selected on LB agar plates containing 25  $\mu$ g/mL chloramphenicol and the presence of ORFble in transformants was confirmed by PCR and sequencing. However, MIC (0.25  $\mu$ g/mL) of zeocin (Thermo Fisher Scientific, Waltham, MA, USA), a bleomycin, against *E. coli* DH5 $\alpha$  transformant containing ORFble was the same as that against DH5 $\alpha$  as

determined using the broth microdilution method (17). This suggests that ORFble did not mediate resistance to bleomycin and its function remains undetermined.

Conjugation experiments were carried out in broth using azide-resistant *E. coli* strain J53 as the recipient and 2 µg/ml colistin plus 150 µg/ml sodium azide was used for selecting transconjugants. The presence of *mcr-I* in transconjugants was confirmed using PCR. In strain WCHKP1511, *mcr-I* could be transferred to *E. coli* J53 at a frequency of  $10^{-2}$  cells per donor cell by mating, suggesting that *mcr-I* was carried by a self-transmissible plasmid, which was assigned pMCR\_1511. The sequence of pMCR\_1511 was completely circularised with gaps between contigs closed by Sanger sequencing of amplicons from PCRs using primers designed based on available contig sequences. pMCR\_KP1511 was 57,278 bp in size and had no known antimicrobial resistance genes other than *mcr-I*. Unlikely the previously-described *mcr-I*-carrying IncI2 plasmid pHNSHP45 (GenBank accession number KP347127) (1), pMCR\_KP1511 belonged to the IncP type, a broad-host-range incompatibility group. Plasmid pKH-457-3-BE carrying *mcr-I* in *E. coli* from Belgium was found to have an IncP backbone (3). However, the sequence of pKH-457-3-BE was not available for further analysis. Nonetheless, it has been suggested that pKH-457-3-BE was 99% similarity and 73% coverage with the IncHI2 plasmid pHXY0908 (GenBank accession number KM877269) in *Salmonella enterica* serotype Typhimurium (8). pMCR\_KP1511 had only a 6% coverage with pHXY0908, indicating that



pMCR\_1511 was very different from pKH-457-3-BE and pKH-457-3-BE may not be a true IncP plasmid but is likely of IncHI2.

pMCR\_1511 has the typical IncP-1 plasmid backbone (23) containing the *trfA* encoding the replication initiation protein, two *par* modules for plasmid partitioning, two conjugative regions *tra* (17.6 kb) and *trb* (12.7 kb), the host-lethal protein-encoding *kil* genes and their regulator *kor* (stands for *kil*-*override*) genes, and a toxin-antitoxin *higA-B* system (Figure 1). The backbone of pMCR\_1511 was highly similar (99% identity) with that on plasmid pHNFP671 (GenBank accession number KP324830), which was an IncP plasmid in *E. coli* isolate FP671 from Guangzhou, China but did not carry *mcr-I*. IncP-1 plasmid has six assigned clades, i.e.,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$ , among which  $\beta$  clade has  $\beta 1$  and  $\beta 2$  two subclades (24). To determine of which clade pMCR\_1511 was, the sequence of 30 genes belonging to the IncP-1 backbone was retrieved from pMCR\_1511, concatenated and then aligned to the counterparts of one representative plasmid of each clade including  $\beta 1$  and  $\beta 2$  subclades as described previously (25). Phylogenetic analysis of the IncP-1 plasmid backbone revealed that pMCR\_1511 and pHNFP671 belonged to a new IncP-1 clade (Figure S1 in the supplementary file).

When compared the sequence of pHNFP671, pMCR\_1511 has two unique regions (Figure 1). One of the unique region harbored *mcr-I* and the other contained ORFble.

153 The sequence comparison of pMCR\_1511 and pHNFP671 allowed us to analyze the  
154 genetic context of *mcr-1* in detail. Like most genetic contexts available in the  
155 GenBank, *mcr-1* was located downstream of the insertion sequence IS*Apl1* on  
156 pMCR\_1511. However, there was another IS*Apl1*, which was interrupted (see below),  
157 downstream and therefore *mcr-1* was bracketed by two copies of IS*Apl1* on  
158 pMCR\_1511. It has been known that IS*Apl1* is able to generate 2-bp direct target  
159 repeats (DR) upon insertion (26)  
160 ([https://www-is.biotoul.fr/index.html?is\\_special\\_name=ISAp11](https://www-is.biotoul.fr/index.html?is_special_name=ISAp11)). The 2-bp flanking  
161 sequences of the region bracketed by the two copies of IS*Apl1* were identical (AC,  
162 Figure 2). When the region formed by two copies of IS*Apl1* and one of the 2-bp  
163 flanking sequences were subtracted artificially, the joined sequence perfectly matched  
164 that of an open reading frame (orf) with unknown function on plasmid pHNFP671. It  
165 therefore proved that the 2-bp sequence was truly DR generated by IS*Apl1* rather than  
166 coincidence and the two IS*Apl1* formed a composite transposon to mobilize *mcr-1*  
167 gene. A very recent analysis revealed that the IS*Apl1*-formed composite transposon  
168 carrying *mcr-1* has also been seen on either the chromosome or a plasmid (IncH or  
169 unknown Inc groups) of seven *E. coli* strains (27). The IS*Apl1*-formed composite  
170 transposon carrying *mcr-1* is all located at different locations, which are also different  
171 from the location on pMCR\_1511, in the seven *E. coli* strains and is flanked by 2-bp  
172 DR in five strains (27). The previous analysis (27) and the findings in the present

study suggest that the IS*ApII*-formed composite transposon is a common vehicle for mediating the spread of *mcr-I*.

The IS*ApII* downstream of *mcr-I* on pMCR\_1511 was interrupted by the insertion of Tn3 with the characteristic 5-bp DR (Figure 2). The Tn3 was also disrupted by IS26 and most of the Tn3 was absent, which may be due to the action of IS26. It is well known that the insertion of IS26 can lead to the deletion of the adjacent sequence of the insertion site (28). Alternative explanation for the absence of most part of Tn3 is that the insertion of the second IS26 and the recombination between the two IS26 could lead to the loss of the intervening region. Although IS*ApII* downstream of *mcr-I* was interrupted by Tn3, the right-end inverted repeat (IRR) of the IS*ApII* remained intact (Figure 2). The transposase encoded by the IS*ApII* upstream of *mcr-I* had the potential to recognize the IRR of the IS*ApII* downstream of *mcr-I* and then could realize the mobilization of the region bracketed by the two copies of the IS*ApII*.

In conclusion, the plasmid-borne colistin resistance gene *mcr-I* was found in a *K. pneumoniae* of an infrequently encountered ST from hospital sewage. *mcr-I* was carried by a self-transmissible IncP plasmid, which is a broad-host-range type of plasmids and has the potential to mediate the dissemination of *mcr-I* from the *Enterobacteriaceae* to other Gram-negative bacteria such as *Pseudomonas aeruginosa*. *mcr-I* was bracketed by two copies of IS*ApII*, which were able to form a composite

transposon and represented a common mechanism for mediating the mobilization of *mcr-1*.

**Nucleotide sequence accession number.** Reads and the Whole Genome Shotgun Sequencing project of *K. pneumoniae* strain WCHKP1511 have been deposited into DDBJ/EMBL/GenBank under accession number SRR3170679 and LSMF000000000, respectively. The sequence of pMCR\_1511 has been deposited into DDBJ/EMBL/GenBank under accession number KX377410.

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## Figure legends

**Figure 1.** Genetic structure of IncP plasmid pMCR\_1511 carrying *mcr-I* and the comparison with IncP plasmid pHNFP671. Regions and genes that are indicated are ORFble (a predicted [but actually not] bleomycin resistance gene), *higA-B* (encoding a toxin/antitoxin system), *mcr-I*, *kor-par-kli* (for plasmid maintenance), *tra* and *trb* (the two conjugation-encoding regions), and *trfA* (encoding the plasmid replication initiation protein). Backbones of the two IncP plasmids are almost identical. Compared to pHNFP671, pMCR\_1511 carried two additional regions that contained either *mcr-I* or ORFble.

**Figure 2.** Genetic context of *mcr-I* on pMCR\_1511. Genetic context of *mcr-I* on the IncI2 plasmid pHNSHP45 (GenBank accession number KP347127) and the corresponding region on the IncP plasmid pHNFP671 (GenBank accession number KP324830) are shown for comparison. orfs that encode hypothetical proteins with unknown function are indicated in white except that the one disrupted by the IS*AplI*-formed composite transposon on pMCR\_1511 is shown in black ( $\Delta 1$  and  $\Delta 2$ ). Other genes shown are *nikB* (encoding relaxase of the plasmid), *ydgA* (DNA topoisomerase III), *ydfA* (transcriptional regulator), *parA* (resolvase), *bla*<sub>TEM</sub> (shown as a white arrow in Tn3) and *traB* (conjugative protein). The 2-bp direct repeat (GA) abutting the IS*AplI*-*mcr-I*-*pho* region on pHNSHP45 and the 2-bp direct repeat (AC) abutting the IS*AplI*-formed composite transposon on pMCR\_1511 are shown. On



328 pMCR\_1511, the IS*AplI* downstream of *mcr-1* was interrupted by Tn3, which was  
329 interrupted by IS26.